**Full Length Research Paper**

**Abutilon avicennae seed coat extract inhibits the lipopolysaccharide-induced inflammatory response in macrophages by multiple mechanisms**

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Abutilon avicennae is used in Korean folk medicine to treat middle ear infection, diarrhea, and inflammation of the lymphatic glands. The anti-inflammatory abilities of abutilon avicennae have not been investigated in detail. Therefore, we undertook the present study to examine whether an extract of the seed coat of abutilon avicennae (SCAA) could suppress LPS-stimulated inflammatory responses in RAW 264.7 cells and to determine the mechanisms by which it did so. We found that SCAA completely suppressed NO production in LPS-stimulated RAW 264.7 cells in a dose-dependent manner. We also confirmed that SCAA was not cytotoxic to RAW 264.7 cells at any of the concentrations we used in this study. Molecular analysis demonstrated that SCAA inhibited mRNA and protein expression of both iNOS and COX-2 in a dose-dependent manner. In addition, SCAA reduced the mRNA levels of the pro-inflammatory cytokines interleukin-1β (IL-1β) and interleukin-6 (IL-6). Moreover, SCAA suppressed the phosphorylation of mitogen-activated protein kinases (MAPKs; JNK, ERK1/2 and p38), whereas it had no effect on the phosphorylation status of Akt. SCAA suppressed the LPS-stimulated degradation of IκBα in a concentration-dependent manner, thereby preventing translocation of nuclear factor-κB (NF-κB) into the nucleus. Taken together, these results suggest that SCAA exerted its anti-inflammatory effects in LPS-stimulated RAW 264.7 cells by inhibiting IκBα degradation, and inhibiting the transcription of proinflammatory mediators and cytokines.

**Key words:** Inflammation, abutilon avicennae, nuclear factor-κB, RAW 264.7.

**INTRODUCTION**

For the reason that traditional herbal medicines are considered to have low toxicity, there has been interest in determining the signaling mechanisms underlying the anti-inflammatory effects of many of these natural products. Abutilon avicennae is used in Korean folk medicine to treat middle ear infection, diarrhea, and inflammation of the lymphatic glands (Park et al., 2001). Macrophages are immune cells that provide an immediate defense against foreign invaders. The expression of inflammatory-involved genes is tightly controlled by several signaling pathways (Ci et al., 2010). Upon activation of the toll-like receptor (TLR) 4 expressed on the surface of macrophages by lipopolysaccharide (LPS), which is an endotoxin present in the cell walls of gram-negative bacteria, several signaling pathways are activated, resulting in the production of inflammatory mediators such as prostaglandin E2 (PGE₂) and nitric oxide (NO) and cytokines such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) (Chen et al., 2010). PGE₂ is synthesized by cyclooxygenase-2 (COX-2), a pivotal enzyme in the inflammatory pathway, while NO is...
generated by nitric oxide synthase (NOS). Cyclooxygenase has been identified to have two major isoforms (COX-1 and COX-2) and one variant (COX-3). COX-1 is present in many tissues, while COX-2 is induced in inflammatory cells such as macrophages (Fang et al., 2011; Francisco et al., 2011). In addition, three isoforms of NOS have been characterized: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Among these isoforms, iNOS catalyzes the production of large amounts of NO from L-arginine in LPS-activated macrophages (Kleinert et al., 2003; Toda et al., 2008).

Extracellular signal-regulated kinase (ERK)1/2, p38, and c-Jun N-terminal kinase (JNK) are signaling molecules in the mitogen-activated protein kinases (MAPK) pathway (Li et al., 2009; Reddy and Reddanna, 2009). The LPS signaling cascade activates the inhibitor of kappa B kinase (IKK) complex, protein kinase B (Akt), and MAPKs (Luyendyk et al., 2008). Akt has also been reported to stimulate the activation of nuclear transcription factor kappa-B (NF-κB) through the phosphatidylinositol-3-kinase (PI3K)-protein kinase B (Akt) pathway in the LPS signaling cascade (Luyendyk et al., 2008; Chaurasia et al., 2010). NF-κB triggers the expression of various inflammatory-related genes. The main inductive form of NF-κB is a heterodimer consisting of the p50/p65 subunits. NF-κB exists as an inactive complex in the cytosol bound to inhibitory protein I kappa B (IkB) in the absence of LPS. In the presence of LPS, IκBα is ubiquitinylated and degraded via phosphorylation by IkB kinase IKK (Baker et al., 2011). Degradation of IκBα allows NF-κB to translocate to the cell nucleus, where it binds to specific DNA sequences and upregulates the expression of target genes involved in inflammation such as iNOS, COX-2, and various cytokines.

In the current study, we investigated the anti-inflammatory effects of an extract from the seed coat of Abutilon avicennae seed coat extracts

Preparation of Abutilon avicennae seed coat extracts

Abutilon avicennae was cultivated according to the good agricultural practices method of the Korea Rural Development Administration and was harvested in 2009 on Eumseong (GPS: E 128° 62’ N 36° 56’). For sample preparation, 400 g of the dried seed coat of abutilon avicennae was extracted three times with 2 L of 95% ethanol at 25°C for three days. The extracts were filtrated through Whatman No. 1 and combined followed by concentration using a rotary evaporator (EYELA N-1000, Japan) at 40°C. The obtained dried extracts of seed coat were 120 g, respectively. The extracts were dissolved in dimethylsulfoxide (DMSO) and added to DMEM with a maximum final DMSO concentration of 0.05%.

Cell culture

RAW 264.7 cells were cultured in DMEM medium supplemented with 10% (v/v) FBS and 100 unit/ml penicillin-streptomycin in an atmosphere of 5% CO₂ at 37°C.

Nitric oxide assay

RAW 264.7 cells (1 × 10⁶ cells/ml) were seeded in a 24-well plate for 24 h at 37°C with 5% CO₂. The culture medium was removed and replaced with fresh medium containing SCAA or DMSO at various concentrations (0, 5, 25, 50 and 100 μg/ml) for 30 min prior to LPS stimulation (100 ng/ml). After 18 h incubation, the quantity of nitrite in the culture medium was measured as an indicator of NO production. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance of each well was measured at 550 nm in a microplate reader (BIOTek Instruments Inc., Winooski, VT, USA). Culture medium was used as a blank in all experiments. The quantification of nitrite was standardized using 0 to 50 μM NaNO₂.

MTT assay to determine cell viability

Cell viability was determined using the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. RAW 264.7 cells (1 × 10⁶ cells/ml) were seeded in a 96-well plate for 24 h at 37°C with 5% CO₂. The medium was then removed and replaced with fresh medium containing various concentrations (0 to 100 μg/ml) of SCAA or DMSO followed by 23 h incubation. Subsequently, MTT solution (5 mg/ml) was added to each well followed by 60 min incubation. The supernatant was removed and the cells were treated with DMSO to dissolve the crystals. After 5 min incubation, the absorbance was read at 595 nm using a microplate reader.

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR) assay

RAW 264.7 cells (1 × 10⁶ cells/ml) were seeded in a 6-well plate for 24 h at 37°C with 5% CO₂. The medium was removed and replaced with fresh medium containing various concentrations (0 to 100 μg/ml) of SCAA or DMSO. After a 30 min incubation, LPS (100 ng/ml) was added to each well followed by an 18 h incubation. The cells were then harvested and washed twice with cold phosphate-buffered saline (PBS, pH 7.2) and total RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions. The concentration of total RNA was determined using a spectrophotometer. One microgram of total

MATERIALS AND METHODS

Materials

The RAW 264.7 macrophage-like cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco’s modified eagle’s medium (DMEM) from Sigma-Aldrich (St. Louis, MO, USA) was used as the cell culture medium. Fetal bovine serum (FBS) and penicillin-streptomycin were obtained from Lonza (Walkersville, MD, USA). LPS was obtained from Sigma-Aldrich (St. Louis, MO, USA). MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) was purchased from USB (Cleveland, OH, USA). Antibodies against iNOS, COX-2, IκBα, phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK, JNK, phospho-Akt, Akt, and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA).
Table 1. Primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>Sense</td>
<td>ATGGCTTGCCCTGGGAAGTTTCTC</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CCTCTGATGGTCGATCGGATCTGT</td>
</tr>
<tr>
<td>COX-1</td>
<td>Sense</td>
<td>GGAGAGACATATCAAGATAGT</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>ATGGTCAGTAGACTTTTACA</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Sense</td>
<td>CAGGATGAGGAGACATGAGCAC</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CTCTGACGACTCAAAGACTCCAC</td>
</tr>
<tr>
<td>IL-6</td>
<td>Sense</td>
<td>GAGGATACCACTCCCAACAGA</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>AAGTGCATCATCTGTGCCCATCA</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense</td>
<td>GTGGGGCGCCCTAGGGCAC</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GGAGGAAGAGGATGCGGAC</td>
</tr>
</tbody>
</table>

RNA was used as a template for cDNA synthesis and PCR was performed using the Accupower RT/PCR premix kit (Bioneer, Daejeon, Korea). The PCR primers used in this study were purchased from Bioneer (Daejeon, Korea) and the primer sequences are provided in Table 1. The PCR cycling profile for amplification of iNOS, IL-6, and IL-1β was as follows: initial denaturation at 42°C for 60 min and 94°C for 5 min; 30 cycles of 94°C for 45 s, 56°C for 45 s, and extension at 72°C for 1 min. For COX-2, the cycling profile comprised an initial denaturation step at 42°C for 60 min and 94°C for 5 min followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, and extension at 72°C for 1 min. A final extension step of 72°C for 10 min was performed for all amplifications. The PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide. PCR products were visualized using a transilluminator (Viabber Lourmat, France).

Protein extraction and western blotting analysis

To extract protein for western blot analysis, RAW 264.7 cells (1 x 10⁶ cells/ml) were seeded in a 6-well plate for 24 h at 37°C with 5% CO₂. The medium was removed and replaced with fresh medium containing various concentrations (0 to 100 µg/ml) of SCAA or DMSO. After a 30 min incubation, LPS (100 ng/ml) was added to each well followed by a 6 h incubation before the levels of iNOS were evaluated. Cells were incubated with LPS for 12 h to measure COX-2 protein expression, 30 min to evaluate iκBα, ERK1/2, and p38 expression, and 45 min to evaluate JNK and Akt expression. The cells were then washed twice with ice-cold PBS (pH 7.2) and collected on ice. The washed cell pellets were centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was removed from the tubes and 50 µl PRO-PREP (iNtRON Biotechnology, Inc., Seongnam, Korea) was added to each tube. The cells were washed three times with TBST buffer and incubated with a secondary anti-rabbit antibody (1:5000) for 1 h at room temperature. The membranes were washed three times with TBST and the proteins were detected using an enhanced chemiluminescence western blotting detection kit (GE Healthcare, UK).

Statistical analysis

Experimental results are expressed as means ± standard deviations. One-way analysis of variance (ANOVA) was followed by Dunnett’s test for multiple comparisons. P-values less than 0.05 or less than 0.01 were considered statistically significant, as indicated.

RESULTS

SCAA inhibited nitric oxide production in LPS-induced RAW 264.7 cells

To investigate the effect of SCAA on NO production in LPS-induced RAW 264.7 cells, the cells were treated with various concentrations of SCAA or DMSO (0, 5, 25, 50 and 100 µg/ml) followed by an 18 h treatment with LPS (100 ng/ml). The amount of nitrite, a stable product of NO released into the culture supernatant, was quantified using Griess reagent. As shown in Figure 1, SCAA dramatically inhibited nitrite production in a dose-dependent manner, whereas LPS treatment significantly increased the concentration of nitrite. To evaluate whether the SCAA-induced decrease in nitrite production was due to cytotoxicity, we evaluated the effect of SCAA on cell viability using the MTT assay. RAW 264.7 cells were treated with various concentrations of SCAA for 23 h. SCAA was not cytotoxic at any of the concentrations examined. Thus, the inhibitory effects of SCAA on NO production were not due to cytotoxicity (Figure 2).
SCAA dose-dependently decreased nitric oxide production in RAW 264.7 macrophages stimulated with LPS. Cells were pretreated with various concentrations of SCAA for 30 min, then with LPS (100 ng/ml), followed by an 18 h incubation. The amount of NO in the culture supernatant was quantified using Griess reagent. The results are an average of 4 similar experiments, expressed by mean ± S.D (* p< 0.05, ** p< 0.01).

**Figure 1.** SCAA dose-dependently decreased nitric oxide production in RAW 264.7 macrophages stimulated with LPS. Cells were pretreated with various concentrations of SCAA for 30 min, then with LPS (100 ng/ml), followed by an 18 h incubation. The amount of NO in the culture supernatant was quantified using Griess reagent. The results are an average of 4 similar experiments, expressed by mean ± S.D (* p< 0.05, ** p< 0.01).

SCAA had no effect on the viability of RAW 264.7 macrophages. Cells were treated with various doses of SCAA. After 23 h incubation, cytotoxicity was evaluated using the MTT assay. The results are an average of 4 similar experiments, expressed by mean ± S.D.

**Figure 2.** SCAA had no effect on the viability of RAW 264.7 macrophages. Cells were treated with various doses of SCAA. After 23 h incubation, cytotoxicity was evaluated using the MTT assay. The results are an average of 4 similar experiments, expressed by mean ± S.D.

SCAA decreased transcript levels of iNOS, COX-2, IL-1β and IL-6 in LPS-stimulated RAW 264.7 cells

Pro-inflammatory cytokines such as iNOS, COX-2, IL-1β and IL-6 play crucial roles in immune responses to various inflammatory stimuli (Luo et al., 2010). Thus, we evaluated transcript levels of iNOS, COX-2, IL-1β and IL-6 using RT-PCR to determine whether SCAA decreased transcription of these genes. Transcription of iNOS, COX-2, IL-1β and IL-6 were remarkably decreased by SCAA under the same conditions (Figure 3). These results indicated that SCAA inhibited
SCAA decreased transcript expression of iNOS, COX-2, IL-6 and IL-1β in LPS-stimulated RAW 264.7 macrophages. Cells were treated with various concentrations of SCAA for 30 min prior to LPS stimulation (100 ng/ml). After 18 h, total RNA was isolated and the transcript levels of iNOS (A), COX-2 (B), IL-6 (C) and IL-1β (D) were determined by RT-PCR analysis. β-actin was used as an internal control. The bands of RT-PCR were analyzed by densitometry and the values are given as the percent of control. The results are an average of 4 similar experiments, expressed by mean ± S.D (* p < 0.05, ** p < 0.01).

SCAA decreased iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells

To characterize the anti-inflammatory activity of SCAA, we determined the effects of SCAA on iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells. LPS treatment alone resulted in a dramatic increase in levels of iNOS and COX-2. In contrast, SCAA decreased the expression of iNOS and COX-2 in a dose-dependent manner (Figure 4).

SCAA inhibited the phosphorylation of ERK1/2, p38, and JNK in LPS-stimulated RAW 264.7 cells but had no effect on the phosphorylation status of Akt

Previous studies have shown that MAPK pathways are involved in the up-regulation of iNOS and COX-2 expression in LPS-activated macrophages (Hiransai et al., 2010; Siebenlist et al., 1994). To elucidate the molecular mechanisms by which SCAA exerted its anti-inflammatory effects in LPS-stimulated macrophages, we examined the effects of SCAA on the phosphorylation status of ERK1/2, p38, JNK, and Akt in LPS-induced RAW 264.7 cells. Western blot analyses with antiphospho-specific antibodies revealed that SCAA suppressed the LPS-induced transcription of inflammatory genes in RAW 264.7 cells.
Figure 4. SCAA decreased protein expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 macrophages. Cells were pretreated with SCAA for 30 min, and then stimulated with LPS. After 6 or 12 h stimulation for iNOS (A) or COX-2 (B), respectively, the cells were lysed and the lysates were examined by western blotting. The bands of western blot were analyzed by densitometry and the values are given as the percent of control. The results are an average of 4 similar experiments, expressed by mean ± S.D (* p< 0.05, ** p< 0.01).

SCAA inhibited IκBα degradation in LPS-induced RAW 264.7 cells

MAPKs modulate NF-κB activity. It is a mammalian transcription factor that is activated by LPS and that regulates the expression of pro-inflammatory enzymes and cytokines such as iNOS, COX-2, IL-6, and IL-1β, as well as cell survival genes (Richmond, 2002). In general, NF-κB is present in the cytoplasm as a complex with IκB, which prevents it from translocating into the nucleus. When macrophages are stimulated with LPS, IκB is phosphorylated, ubiquitinated, and subsequently degraded, allowing NF-κB to translocate to the nucleus (Karin and Men-Neriah, 2000). To analyze whether SCAA had an impact on the degradation of IκBα, cells were treated with SCAA for 30 min followed by stimulation with LPS for 30 min. As shown in Figure 6, SCAA attenuated the LPS-induced degradation of IκBα in a dose-dependent manner. These results suggest that SCAA therefore appears to prevent LPS-induced NF-κB activation by inhibiting IκBα degradation in RAW 264.7 cells.

DISCUSSION

In the process of inflammation, macrophages provide an immediate defense against foreign stimuli such as LPS (Kim et al., 2010). In response to LPS stimulation, macrophages generate a variety of cytotoxic-inflammatory mediators including nitric oxide, PGE2, and various inflammatory cytokines (Mosser and Edwards, 2008). However, excessive release of these molecules can result in various inflammatory disorders, which has stimulated interest in determining the intercellular signaling pathways and molecules involved in inflammatory responses (Park et al., 2007; Lewis and Manning, 1999).

In activated macrophages, NO, an important pro-inflammatory regulator, is generated from L-arginine by iNOS (Grisham et al., 1999). Although NO has been demonstrated to have beneficial microbicidal, antiviral, immunomodulatory, and antitumoral effects, high amounts of NO are implicated in the pathophysiology of several human diseases (Bogdan, 2011; Guzik et al., 2003). In our search for plant-based anti-inflammatory compounds, we found that a SCAA had an anti-inflammatory effect in LPS-induced RAW 264.7 cells. We therefore examined the effects of SCAA on pro-inflammatory mediators and cytokines in RAW 264.7...
Figure 5. SCAA inhibited the phosphorylation of ERK1/2, p38, and JNK in LPS-stimulated RAW 264.7 macrophages. Cells were pre-incubated with SCAA for 30 min prior to stimulation with LPS for 30 min (for ERK1/2 and p38) or 45 min (for JNK and Akt). Total cell lysates were then obtained and examined by western blotting analysis using the antibodies indicated in the figure. The bands of western blot were analyzed by densitometry and the values are given as the percent of control. The results are an average of 4 similar experiments, expressed by mean ± S.D.

Figure 6. SCAA prevented I\(\kappa\)B\(\alpha\) degradation in LPS-induced RAW 264.7 macrophages. Cells were treated with SCAA for 30 min followed by stimulation with LPS for 30 min. Total cell lysates were then obtained and western blotting analysis of I\(\kappa\)B\(\alpha\) levels was performed. The bands of western blot were analyzed by densitometry and the values are given as the percent of control. The results are an average of 4 similar experiments, expressed by mean ± S.D (* p< 0.05, ** p< 0.01).
macrophages activated by LPS to determine the mechanism(s) by which SCAA inhibits inflammation.

iNOS and COX-2, which are inducible enzymes, play critical roles in inflammation, cell proliferation, and skin tumor promotion (Murakami and Ohigashi, 2007; Chang et al., 2002). iNOS and COX-2 catalyze the synthesis of NO and prostaglandins, respectively. Also, pro-inflammatory cytokines, such as IL-1β and IL-6 are important mediators of acute and chronic inflammatory disorders. Using RT-PCR, we found that SCAA decreased the expression of iNOS, COX-2, IL-1β and IL-6 transcripts (Figure 3). We also found that SCAA dose-dependently inhibited the expressions of iNOS and COX-2 at the protein level (Figure 4).

The over-expression of these enzymes is regarded as an initiating factor in various inflammatory diseases (Kanwar et al., 2009). A previous study reported that monoterpenoid D-limonene had an anti-inflammatory effect on the production of NO, PGE2, and pro-inflammatory cytokines in RAW 264.7 macrophages (Yoon et al., 2010). In another report, tormentic acid isolated from Rosa rugosa was found to have an anti-inflammatory effect in RAW 264.7 cells by down-regulating iNOS and COX-2 expression (An et al., 2011).

NF-κB is a ubiquitous transcription factor that regulates the expression of a variety of genes involved in chronic inflammatory diseases (Pan et al., 2011). In resting macrophages, NF-κB is bound to IκB, rendering this protein inactive. After LPS activation, IKKs phosphorylate IκB proteins, target them for ubiquitination and proteasomal degradation. These releases NF-κB, which then translocates to the nucleus and binds to NF-κB binding sites to initiate the transcription of several target genes including iNOS, COX-2, IL-1β, IL-6, and TNF-α (Nau et al., 2002). We found that SCAA inhibited IκB degradation in a concentration-dependent manner in LPS-induced RAW 264.7 cells (Figure 6). SCAA therefore exerts its anti-inflammatory effects by stabilizing IκBα, thereby blocking nuclear translocation of NF-κB.

The activation of NF-κB is also controlled by cellular kinases such as MAPKs (Oh et al., 2008). ERK1/2, p38, and JNK are signaling molecules downstream of MAPKs. MAPKs, a family of serine/threonine protein kinases, respond to extracellular signals and activate intracellular signaling pathways. In addition, MAPKs regulated the transcriptional activity of the transcription factor AP-1, which binds to specific nucleotide sequences with NF-κB, thereby triggering the transcription of inflammatory-related downstream genes (Manarin et al., 2010). We therefore examined the effects of SCAA on the phosphorylation status of MAPKs in RAW 264.7 macrophages stimulated with LPS. SCAA dose-dependently suppressed the phosphorylation of ERK1/2, p38, and JNK (Figure 5). These results imply that SCAA suppresses NF-κB activity by inhibiting ERK1/2, p38, and JNK phosphorylation in LPS-stimulated RAW 264.7 cells.

In summary, we found that SCAA had a significantly anti-inflammatory effect in LPS-induced RAW 264.7 cells by inhibiting iNOS expression and therefore NO production as well as down-regulating the expression of COX-2 and other pro-inflammatory mediators such as IL-1β and IL-6. The SCAA-induced down-regulation in expression of these pro-inflammatory proteins was due to suppression of NF-κB activity by inhibition of IκBα phosphorylation. SCAA also inhibited the phosphorylation of MAPKs that activate a variety of inflammatory-involved factors. SCAA therefore shows promising potential as an anti-inflammatory drug.

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REFERENCES


